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Evaluation of the microbiome of decaying alder nodules by next generation sequencing

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This work investigated the microbial content of decaying nodules from alders. The 16S *rDNA* composition of the microbiome of six senescent alder nodules was investigated by 454 sequencing. All nodules still had some Frankia sequences present, but in each case it was only detected at minor levels, with other organisms predominating. Although organisms from three different phyla (Bacteroidetes, Proteobacteria and Actinobacteria) constituted almost all (98% or more) of all sequences, Bacteroidetes were most abundant in four nodules with Proteobacteria being most abundant in the other two. In addition a few families were represented at a level of 10% or more of the total sequences: Sphingobacteriaceae (all 6 nodules); Chitinophagaceae (5 of 6); non-Frankia Actinomycetales (2 of 6); Caulobacteraceae (2 of 6); Flavobacteriaceae (2 of 6); Oxalobacteraceae (1 of 6); and Xanthomoadaceae (1 of 6). Analysis at the genus level showed a diverse range of organisms, with members of the genus Pedobacter being found at an abundant level within most nodules.

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Introduction

Members of the genus Frankia are actinomycetes which fix atmospheric nitrogen in symbiotic association with the roots of a range of trees and woody shrubs, including alders. In this partnership Frankia acts as the microsymbiont, with the host plants acting as the macrosymbiont. As a result of this relationship the trees show an improved growth due to the provision of fixed nitrogen. In addition, other organisms also present in the immediate environment may also gain benefits in terms of access to nitrogenous compounds, which is the reason that underpins the use of alder trees to help facilitate growth of other trees in forestry practices. By way of illustration, many species of alders (e.g. *Alnus glutinosa*, *A. rubra*) have been planted in forestry plots to facilitate growth of the trees planted with them (Wheeler and Miller 1990) due to fixation of atmospheric nitrogen taking place in their root systems.

Mature alder trees will commonly have several nodules associated with their root systems, and it is known that actinorhizal plants can have more than one strain of Frankia in the various nodules around their roots (Dobritsa and Stupar 1989). However the level of diversity within an alder single nodule is low (Kennedy et al. 2010), with each nodule containing one predominant strain (Dai et al. 2004; McEwan et al. 2015).

There has been considerable research carried out into the nodulation process (e.g. Berry et al. 1986; Van Ghelue et al. 1997) and also the events within the nodule during the process of nitrogen fixation (e.g. Vikman et al. 1990) including analysis of genes involved in nitrogen-fixation (e.g. Normand et al. 1988). However, although there has been some work done to examine what happens after nodule senescence, the level of information is relatively limited. To date, it is known that a loss of nitrogenase activity in the microsymbiont is one of the first signs of nodule senescence in both alders (Vikman et al. 1990) and legumes (Swaraj et al. 1993). In terms of the macrosymbionts it has been shown that the alder protein *ag13* may act as a plant marker for senescence (Guan et al. 1997) and that in the case of soybeans cystatins increase during the onset of senescence (van Wyk et al. 2014).

However there has been no work done to investigate the bacterial species which invade a senescing alder nodule, and presumably obtain the benefits of nutrients from a niche rich in fixed nitrogen. The work presented here is the first

application of next generation sequencing to investigate the microbiome of the bacterial community within senescing nodules from *A. glutinosa* roots by analysing amplicons produced from PCR using 16S *rDNA* primers.

Materials and Methods

Collection of nodules

Root nodules were collected from *A. glutinosa* plants which had been growing at the Henfaes Experimental Station, Abergwyngregyn, Gwynedd, Wales (53°14'N, 4°01'W) for 3 years. The nodules were harvested from approximately 5 cm below the soil surface and only those already showing early signs of senescence were used for the current analysis. Following collection, nodules were stored in a freezer at -80°C until ready for DNA analysis.

Performing PCR on nodule material

Thawed nodules were surface-washed to remove the nodule's surface-associated bacteria, taking care to avoid damaging the internal regions of the nodular structure. The periderm was peeled to remove the outer layer from six nodules, each from a different tree. An individual lobe from each nodule was used for extraction of DNA and subsequent analysis.

Lobes were placed in sterile molecular-grade water and then ground in microfuge tubes with mini-pestles, followed by a 15 min incubation at 95°C and centrifugation at 13000 *g* for 10 min. Supernatants were removed and 1 µl of it was added directly to a FastStart high fidelity PCR system master mix. This approach of performing PCR directly on extracted material has been applied previously to Frankia analysis (McEwan and Wheeler 1995; McEwan et al. 2015) with the 95°C incubation rupturing even relatively robust organisms. The following PCR conditions were performed in keeping with the guidelines of the manufacturer (Roche) for FastStart PCR for 454 sequencing of 16S *rDNA* amplicons: 95°C, 2 min followed by 30 cycles of 95°C for 30 s, 55°C for 30 s and 72°C for 2 min and a final extension of 72°C for 7 min. Primers used were barcoded prokaryote primers (Caporaso et al. 2010) and were used to target the V1-V3 region of the bacterial 16S *rRNA* genes. One primer was used as the forward primer in all reactions (CCTATCCCCTGTGTGCCTTGGCAGTCTCAGAGAGTTTGATCM TGGCTCAG), corresponding to *Escherichia coli* position 27. Details of the reverse primers used are contained in Table 1. By using a different reverse primer for each of the six samples allowed identification of the source nodule. In all cases these corresponded to *E. coli* position 357. A final primer concentration of 400 nM was used with a reaction volume of 25 µl. After PCR was completed, amplicons were checked by electrophoresis on a 1% TAE agarose gel to verify successful amplification and that amplicons were around the approximate size predicted when using these primers (i.e. 300 bp).

Sample quantification was carried out using the Quant-iT™ PicoGreen® dsDNA reagent (Invitrogen) and a CFX 96™ real-time system (Bio-rad) to measure relative fluorescence. Concentrations were calculated from a standard curve and reactions were normalized and pooled. Samples were normalized by mixing the six PCR products in equimolar concentrations. All replicates within the mix went through a final concentration measurement and samples were stored at -20ºC until ready for 454 sequencing. Unincorporated dNTPs and primer dimers were removed using Agencourt® AMPure® XP beads (Beckman Coulter). Following this step, the pooled and purified libraries were re-quantified and diluted to a final concentration of 10^7 molecules/µl.

Table 1: Reverse primers used in the current work. All primers corresponded to *E. coli* position 357.

Nodule	Primer sequence (5' to 3')
1	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGAGTGCGTCTGCTGCCTYCCGTA
2	CCATCTCATCCCTGCGTGTCTCCGACTCAGAGCACTGTAGCTGCTGCCTYCCGTA
3	CCATCTCATCCCTGCGTGTCTCCGACTCAGATCAGACACGCTGCTGCCTYCCGTA
4	CCATCTCATCCCTGCGTGTCTCCGACTCAGATATCGCGAGCTGCTGCCTYCCGTA
5	CCATCTCATCCCTGCGTGTCTCCGACTCAGCGTGTCTCTACTGCTGCCTYCCGTA
6	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTCGCGTGTCCTGCTGCCTYCCGTA

Sequencing and bioinformatical analysis

DNA sequencing was performed using a Genome Sequencer FLX, 454 Life Sciences (Roche) with subsequent data analysis being performed on a Dell PowerEdge T710 with Biolinux Ubuntu 10.04 base running QIIME version 1.6.0 (Caporaso et al. 2010). De-replication of the raw input sequences was performed according to the barcode identifiers. Quality control was carried out using the default setting for minimum quality score, length, ambiguous bases, homopolymer runs and primer mismatches. The various operational taxonomic units (OTUs) were selected using UCLUST (Edgar 2010) at identity levels of 97% and the Ribosomal Database Project (RDP) (Cole et al. 2005) was used to assign taxonomy. For all samples, Good's coverage and rarefaction curves were determined using rich (a package run in R) for samples to assess if the number of sequences appeared to as an appropriate representation of the population (Rossi 2011).

The top three most abundant bacterial sequences for each nodule were identified using a BLASTn search against the NCBI nucleotide database (Altschul et al. 1990) at https://blast.ncbi.nlm.nih.gov/Blast.cgi.

Results

Sequences obtained were deposited in the Genbank database with the accession codes ERS1732949, ERS1732952, ERS1732953, ERS1732954, ERS1732955 and ERS1732956.

In all six nodules trace levels (<1% of all sequences) of Frankia were identified, with the majority of sequences being from other organisms.

The non-Frankia sequences were classified at the level of phylum, class and order. The percentage of each taxonomic sequence group relative to the total number of sequences per nodule is shown in Table 2. In four of the six nodules the most abundant phylum was Bacteroidetes, where organisms from this phylum constituted more than half of the sequences detected, with sequences from the phylum Proteobacteria being second most abundant. In the other two nodules sequences belonging to the phylum Proteobacteria were most abundant, comprising more than half of the sequences in one example and almost half in the other, with sequences belonging the phylum Bacteroidetes being second most abundant. The only two other phyla detected in any of the nodules were Acidobacteria (present in all nodules and always third most abundant) and Firmicutes (five of six nodules and never more than 1.5% of the total number).

Table 2: The percentage of different phyla, classes and orders of sequences present in each nodule

Classification	Nodule 1	Nodule 2	Nodule 3	Nodule 4	Nodule 5	Nodule 6
Acidobacteria	2.4	6.8	18.5	11.7	7.3	8.3
Acidobacteria_Gp1	-	0.1	-	-	-	0.5
Granulicella	-	0.1	-	-	-	0.5
Actinobacteria	2.4	6.8	18.5	11.7	7.3	7.8
Actinomycetales	2.4	6.8	17.3	11.3	7.3	7.8
Solirubrobacterales	-	-	1.2	0.4	<0.1	-
Bacteroidetes	85.5	73.8	38.1	23.0	59.5	60.2
Flavobacteriia	-	11.8	15.0	7.0	29.4	5.3
Flavobacteriaceae	-	11.8	15.0	7.0	29.4	5.3
Sphingobacteriia	85.5	62.0	23.1	16.0	30.1	54.9
Chitinophagaceae	0.1	4.7	0.7	0.5	7.5	7.3
Cytophagaceae	-	1.5	3.3	3.6	2.5	0.8
Sphingobacteriaceae	85.4	55.8	19.1	11.9	20.1	46.8
Firmicutes	0.1	0.1	-	0.9	1.5	0.1
Bacilli	0.1	0.1	-	0.9	1.5	0.1
Bacillaceae 2	0.1	-	-	-	-	-
Paenibacillaceae 1	-	0.1	-	0.4	1.5	0.1
Thermoactinomycetaceae 1	-	-	-	0.4	<0.1	-
Proteobacteria	12.0	19.3	43.4	64.4	31.7	31.5
Alphaproteobacteria	3.6	7.8	9.8	43.7	25.6	10.2
Acetobacteraceae	<0.1	-	-	-	-	0.1
Beijerinckiaceae	<0.1	<0.1	-	-	-	<0.1
Bradyrhizobiaceae	1.4	0.8	0.2	0.5	0.4	1.6
Brucellaceae	<0.1	-	-	-	-	-
Caulobacteraceae	-	0.2	0.7	25.7	12.5	0.1
Hyphomicrobiaceae	0.1	0.5	6.5	10.9	10.0	0.7
Methylocystaceae	<0.1	0.1	<0.1	-	0.1	0.1
Phyllobacteriaceae	1.0	1.0	0.3	3.9	0.8	1.1
Rhizobiaceae	0.8	0.8	0.8	1.8	0.2	1.7
Rhizomicrobium	-	0.2	-	-	-	0.3
Rhodobacteraceae	-	0.1	-	-	-	0.1
Sphingomonadaceae	0.2	4.1	1.3	0.9	1.6	4.4
Betaproteobacteria	1.4	9.1	4.8	14.9	3.2	14.0
Alcaligenaceae	-	-	-	0.6	0.4	-
Burkholderiaceae	0.2	<0.1	-	-	-	<0.1
Comamonadaceae	0.8	4.1	4.2	1.5	1.5	5.8
Methylophilaceae	-	0.1	-	-	-	2.4
Oxalobacteraceae	0.4	4.9	0.7	12.7	1.1	5.8
Rhodocyclaceae	-	-	<0.1	0.1	0.2	-
Gammaproteobacteria	7.1	2.3	28.8	5.9	2.8	7.3
Pseudomonadaceae	2.2	0.6	1.9	1.0	1.3	4.4
Xanthomonadaceae	4.8	1.7	26.9	4.9	1.5	2.9

Two bacterial classes were detected within the Bacteroidetes; Flavobacteriia and Sphingobacteriia, with Sphingobacteriia being the more abundant in five of the six nodules, with them being present in approximately equal numbers in the other. Of the sequences identified as being from the class Sphingobacteriia the majority (66% or more) were from the order Sphingobacteriiaceae.

Proteobacteria were represented by three different classes in all six nodules (Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria), with the major class varying from nodule to nodule. Nodules three and four had the highest relative abundance of Proteobacteria, with Alphaproteobacteria more abundant in nodule four (43.7% of all sequences) and Gammaproteobacteria more abundant in nodule three (28.8% of all sequences).

In terms of non-Frankia members of the phylum Acidobacteria, the majority (>90%) of the sequences identified were members of the order Actinomycetales in all nodules.

In all cases the abundance of Firmicutes was relatively low with all sequences assigned as members of the class Bacilli. Table 3 shows the number of operational taxonomic units (OTUs) detected per nodule for each of the classification levels. It is clear that even at the level of different orders, there is representation by many different organisms.

Table 3: The number of distinct sequences present for each phylum, class and order present in each nodule. Names underlined denote the same single member of a taxon was the only one identified in any of these nodules.

Classification	Nodule 1	Nodule 2	Nodule 3	Nodule 4	Nodule 5	Nodule 6
Acidobacteria	12	25	29	23	24	23
Acidobacteria Gp1	0	1	0	0	0	1
Granulicella	0	1	0	0	0	1
Actinobacteria	12	24	29	23	24	22
Actinomycetales	12	24	26	20	23	22
Solirubrobacterales	0	0	3	3	1	0
Bacteroidetes	10	27	21	23	25	30
Flavobacteriia	0	5	5	8	7	6
Flavobacteriaceae	0	5	5	8	7	6
Sphingobacteriia	10	22	16	15	18	24
Chitinophagaceae	1	7	5	5	4	7
Cytophagaceae	0	1	1	1	1	1
Sphingobacteriaceae	9	14	10	9	13	16
Firmicutes	1	2	0	3	3	1
Bacilli	1	2	0	3	3	1
Bacillaceae 2	1	0	0	0	0	0
Paenibacillaceae 1	0	2	0	2	2	1
Thermoactinomycetaceae 1	0	0	0	1	1	0
Proteobacteria	29	47	34	38	39	50
Alphaproteobacteria	20	32	21	21	23	34
Acetobacteraceae	1	0	0	0	0	1
Beijerinckiaceae	1	1	0	0	0	1
Bradyrhizobiaceae	5	7	3	4	4	6
Brucellaceae	1	0	0	0	0	0
Caulobacteraceae	0	4	3	3	4	2
Hyphomicrobiaceae	2	3	4	4	3	3
Methylocystaceae	1	1	1	0	1	1
Phyllobacteriaceae	4	3	2	2	3	4
Rhizobiaceae	3	3	1	2	1	3
Rhizomicrobium	0	1	0	0	0	1
Rhodobacteraceae	0	1	0	0	0	1
Sphingomonadaceae	2	8	7	6	7	11
Betaproteobacteria	4	7	5	8	9	8
Alcaligenaceae	0	0	0	1	1	0
Burkholderiaceae	1	1	0	0	0	1
Comamonadaceae	2	1	1	4	3	2
Methylophilaceae	0	1	0	0	0	1
Oxalobacteraceae	1	4	3	2	4	4
Rhodocyclaceae	0	0	1	1	1	0
Gammaproteobacteria	5	8	8	9	7	8
Pseudomonadaceae	2	1	2	2	2	1
Xanthomonadaceae	3	7	6	7	5	7

The three most abundant taxa in each nodule are listed in Table 4. Pedobacter spp. were among the top three species in four of the six nodules, and in two of these nodules, all three of the most abundant sequences were associated with different organisms from this single genus. Other than this, the most abundant species were identified as Brevundimonas sp., Chryseobacterium sp., Devosia riboflavin, Flavobacterium sp., Massilia sp., Rhodanobacter sp., *Stenotrophomonas rhizophila* and *Streptomyces prunicolor*.

Table 4: The identity of the three most abundant sequences in each of the nodules

Nodule	Most abundant	2nd most abundant	3rd most abundant
1	*Pedobacter panaciterrae*	*Pedobacter humicola*	*Stenotrophomonas rhizophila*
2	Pedobacter sp.	*Pedobacter panaciterrae*	Pedobacter sp.
3	Rhodanobacter sp.	*Streptomyces prunicolor*	*Flavobacterium* sp.
4	Brevundimonas sp.	Massilia sp.	*Devosia riboflavina*
5	Chryseobacterium sp.	Brevundimonas sp.	Pedobacter sp.
6	*Pedobacter panaciterrae*	Pedobacter sp.	Pedobacter sp.

Discussion

The first point to make is that although these nodules were all identified as showing signs of early senescence, they were not selected as being at the same stage in all cases. Therefore the current work does not necessarily identify the initial colonisers, but rather serves as a guide to the types of organisms which are opportunistic in terms of invading the nodules at an early stage after their physical appearance has changed from the form shown by those which are actively fixing nitrogen.

In all cases low levels (<1% of all sequences) were identified as being Frankia. This is in contrast to the situation reported in functional nodules (McEwan et al. 2015) where the majority of the sequences were from a single strain of Frankia. Likewise in most cases a low level of chloroplast sequences from *A. glutinosa* (the macrosymbiont plant in this relationship) were also detected (data not presented). Similar to the Frankia content, this was always less abundant than that detected in the functional nodules.

In the case of the other sequences detected, the composition varied from nodule to nodule, suggesting that there is no distinct pattern to be seen, and that decaying nodules can be invaded by whatever opportunistic organisms may be in a particular environment at any one time. However, it is interesting to note that members of the genus Pedobacter were detected among the three most abundant OTUs in four of the nodules (see Table 3), and in two cases all three of the most abundant OTUs were Pedobacter sequences. Moreover, even the two which did not have Pedobacter sequences among the three most abundant OTUs still had sequences which were identified at low levels in the nodules (data not presented). In total four different OTUs corresponding to this genus were identified; *Pedobacter humicola* (Dahal and Kim 2016), *Pedobacter panaciterrae* (Yoon et al. 2007) and two unclassified members of the genus Pedobacter. Members of the genus Pedobacter (phylum Bacteroidetes, class Sphingobacteriia, order Sphingobacteriaceae) are Gram-negative rods which are often detected in soil samples (Margesin and Shivaji 2015). Members of this genus have been implicated as having a role to play in terms of microbial mediation of components of the nitrogen cycle such as nitrification and denitrification (e.g. Harter et al. 2016).

Other relatively abundant organisms identified in Table 3 were: *Stenotrophomonas rhizophila* (Wolf et al. 2002); *Streptomyces prunicolor* (Shirling and Gottlieb 1969); Rhodanobacter sp. (Nalin et al. 2015); Flavobacterium sp. (Bernardet and Bowman 2015); Brevundimonas sp. (Vancanneyt et al. 2015); Massilia sp. (Editorial Board 2015); *Devosia riboflavin* (Nakagawa et al. 1996) and Chryseobacterium sp. (Bernardet et al. 2015). All of these have been described as being organisms which are found in general environmental organisms found in many ecosystems, with most of them being regarded as being found in either soil or silt samples.

Thus it is clear that there is no obvious single pattern regarding the species associated with early colonising of decaying nodules, it appears that members of the genus Pedobacter are among the more abundant organisms present, with others being representative of those likely to be found in the general rhizosphere at that time.

Conclusion

As alder nodules start to decay, there is no one group of bacteria which are first to colonise the nodules.

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